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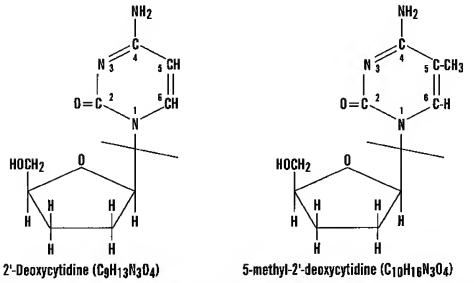
(54) Title: A METHOD TO ASSESS GENOMIC DNA METHYLATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTOSPRAY IONIZATION MASS SPECTROMETRY



CHEMICAL STRUCTURE 2'-deoxycytidine AND 5-methyl-2'-deoxycytidine RESPECTIVELY. CYTOSINE BASE ISOTOPE-LABELED COMPOUNDS CAN BE CREATED BY SUBSTITUTING ANY N WITH ^{15}N, OR H WITH *d*, OR C WITH ^{14}C OR O WITH ^{18}O, IN ANY POSSIBLE COMBINATION.

(57) Abstract: The present invention provides a method for quantitative determination of 5-methyl-2'-deoxycytidine in human DNA using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). The method comprises the steps of enzymatically hydrolyzing the DNA sample by sequential digestion with enzymes; separating the DNA hydrolyzates by reverse-phase high-performance liquid chromatography in isocratic mode wherein the four major DNA bases and 5-methyl-2'-deoxycytidine are resolved and eluted; identifying the 2'-deoxycytidine and 5-methyl-2'-deoxycytidine by combining diode array UV spectra analysis and mass spectra of chromatographic peaks. The isotopomes $^{15}N_3$ 2'-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine are used as internal standards. Ions of *m/z* 126 and 130 are used to detect 5-methyl-2'-deoxycytidine and its isotopomer, and ions of *m/z* 112 and 115 are used to detect 2'-deoxycytidine and its stable isotopomer, respectively. The DNA methylation status is consequently calculated based on the amount of 5-methyl-2'-deoxycytidine per μg DNA with percent relative standard deviations (%RSD) for method precision of 7.1 (within-day) and 5.7 (day-to-day). The method of the present invention also allows the measurement of 5-methyl-2'-deoxycytidine expressed as a percentage of total deoxycytidine residues in genomic DNA with % RSD for method precision of 1.9 (within-day) and 1.7 (day-to-day). The LC/MS method for quantitative determination of genomic DNA methylation status is rapid, sensitive, selective and precise.

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A METHOD TO ASSESS GENOMIC DNA METHYLATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

[001] This invention was made with government support under agreement no. 58-1950-9-001 awarded by the U.S. Dept. of Agriculture. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[002] In most organisms, genetic material is composed of deoxyribonucleic acid ("DNA"), the primary structure of which codes for the amino acid sequence of proteins in the organism. DNA consists of two intertwined polynucleotide chains, each comprising a string of nucleic acid bases linked together by a sugar-phosphate linkage. In a protein-encoding DNA segment, or "gene," the sequence of the protein is determined by the sequence of four types of bases in the DNA: adenine(A), guanine (G) , cytosine (C), and thymine (T). The "expression" of a gene, resulting in the production of an encoded protein, involves transcribing the DNA of the gene into a nucleic acid intermediate called "messenger ribonucleic acid" (mRNA). The mRNA has the same nucleotide base sequence as the DNA from which it is transcribed, except the mRNA contains another base, uracil (U), in place of thymine.

[003] Regulation of gene expression in a cell is what distinguishes one cell type from another. During cellular differentiation, a cell-type-specific pattern of gene expression is established via complex interactions that can involve, for example, extracellular signals and tissue-specific transcription factors.

[004] A characteristic feature of many eukaryotic genomes is methylation of cytosine at the carbon 5' position of CpG dinucleotides.[1] Typically the methylation of cytosine occurs predominantly in CpG rich regions, the so-called 'CpG islands' that are largely localized in gene promoter regions or in the initial exons of genes.[2] DNA methylation is a fundamental mechanism for epigenetic control of gene expression and the maintenance of genomic integrity.[3-5] Therefore, evaluation of genomic DNA

methylation status is critical for the study of cell growth regulation, tissue specific differentiation, [1,2,6] and carcinogenesis.[5]

[005] The two most widely used methods for assessing genomic DNA methylation status are, a Southern blot technique that follows digestion with methylation-sensitive restriction endonucleases [1,7-10] and a radioassay that utilizes a bacterial DNA methyltransferase to catalyze the *de novo* methylation of the cytosine-guanine doublet sites with a radioactive methyl donor *in vitro*.[11-15] Recently another method has been described based on methylation-sensitive endonucleases followed by single nucleotide extension with radiolabeled [^{3}H]dCTP.[16] Generally, these methods have wide variations in precision due to inconsistencies in the activity of methyl sensitive endonucleases, and due to the instability of methyltransferase activity.[17]

[006] Chromatography has been used for the separation of purines and pyrimidines[18] as well as for the identification of modified deoxyribonucleosides.[19-23] Reverse-phase high performance liquid chromatography (HPLC) methods have been also applied for genome-wide methylation analysis. These HPLC methods, however, require the availability of microgram quantities of genomic DNA (5-50 μg), and synthesis of ^{32}P-labeled deoxyribonucleosides, as well as a relatively long run time.[19,21]

[007] In 1976, a mass spectrometry technique was described to obtain higher sensitivity in the detection and identification of 5-methylated cytosine residues from intact underivatized DNA.[24] Thereafter, in the 1980s a derivatization method using N-methyl-N-(t-butyl)dimethylsilyl)trifluoroacetamide for the detection of 5-methylcytosine by stable isotope dilution GC/MS using deuterium labeled 5-methylcytosine, was described for the analysis of modified bases in calf thymus DNA.[25,26] Separation and detection of 5-methylcytosine was also obtained after derivatization with trifluoroacetic anhydride.[27] A further study described the use of HPLC combined with GC/MS [28] for the achievement of better specificity for the identification of the free DNA bases following derivatization with bis(trimethylsilyl)-trifluoroacetamide.[28,29] The application of GC/MS to DNA hydrolyzates has been limited, however, due to the intrinsic polarity of these compounds.[30]

[008] The development of electrospray ionization (ESI) enabled Liquid Chromatography/Mass Spectrometry (LC/MS) to be utilized for the quantitative

determination and structural characterization of a great number of polar/ionic molecules in biological samples, such as nucleic acids.[31-39] The use of LC/MS was suggested recently for the assessment of genomic methylation in DNA purified from a green alga.[40] This procedure, however, requires the availability of a rather large amount of DNA (25 μg), ^{32}P labeling of individual nucleosides, and uses an off-line combination of HPLC and MS, [40] in which the HPLC is used to separate and identify the deoxyribonucleosides by retention times and ultraviolet (UV) absorbance, and the ESI-MS [34] is used to analyze individually the compounds collected as HPLC fractions.[40] The present invention addresses the need for a more efficient, quantitative measurement of DNA methylation.

SUMMARY OF THE INVENTION

[009] The present invention provides a new, on-line LC/MS method for the measurement of methylated cytosine residues in genomic DNA, including:

[0010] isolating genomic DNA,

[0011] hydrolyzing the genomic DNA to form DNA hydrolyzates,

[0012] adding a stable cytosine base isotope to the DNA hydrolyzates,

[0013] subjecting the DNA hydrolyzates to reverse-phase high performance liquid chromatography,

[0014] identifying the products in step (d) by electrospray ionization enabled mass spectrometry, and

[0015] quantitating DNA methylation.

[0016] The present LC/MS method allows the quantitative determination of genomic DNA methylation status. The technique relies on the quantitative hydrolysis of DNA, if necessary the complete removal of potential residual RNA, and enables the separation and identification of the DNA bases and 5-methyl-2'-deoxycitidine by ESI-MS. Furthermore, the quantity of DNA utilized in this method is relatively low and the on-line LC/MS method has a shorter run time for each sample compared to previously described methodologies. The present invention enables analysis of large sample sets that could not be confidently analyzed with previous methods.[17] Since the determination of DNA methylation is crucial for the study of gene expression and stability, the applicability of this method extends to a wide field of biochemistry and molecular biology studies.

[0017] In accordance with the present invention, genomic DNA is isolated by classical methods, e.g. phenol:chloroform:isoamyl alcohol, from sources including tissue and cells, as well as cultured cells. The DNA is hydrolyzed using standard methods including enzymatic digestion. Residual RNA may then be removed using RNase. Methylated and/or unmethylated stable cytosine base isotope-labeled compounds, e.g. $^{15}N_3$-2'-deoxycytidine and/or the custom-made methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine, are then added as internal standards for 2'-deoxycytidine and/or 5-methyl-2'-deoxycytidine residues, respectively. Other stable cytosine base isotope-labeled compounds may be used provided they have the general form of deoxycytidine and methyl-deoxycytidine (see Figure 1), wherein N may be ^{14}N or ^{15}N, O may be ^{16}O or ^{18}O, C may be ^{13}C or ^{14}C and H may be ^{1}H or ^{2}H, in any combination. Preferably, isotope substitution is done on the pyrimidine ring, rather than on the pentose. During the electrospray ionization, the sugar generally separates from the pyrimidine. The sugar alone cannot be used for analysis because it is identical in Cyt and mCyt. However, a protocol in which a part of the sugar is analyzed may be used.

[0018] The DNA hydrolyzates are separated by reverse-phase high performance liquid chromatography in isocratic mode or solution gradient mode, etc. Isocratic mode is preferable as it allows the same eluent to be used throughout the separation. Identification of 2'-deoxycytidine and 5-methyl-2'-deoxycytidine is obtained by combined diode array UV spectra analysis and mass spectra of chromatographic peaks using a mass spectrometer equipped with an electrospray ionization source. The DNA methylation status is then calculated based on the amount of 5-methyl-2'-deoxycytidine per μg of DNA.

[0019] The LC/MS method of the present invention allows accurate measurement of the absolute amount of 5-methyl-2'-deoxycytidine in DNA by utilizing a newly synthesized methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine stable isotopomer. The use of the isotope-labeled compound $^{15}N_3$ 2'-deoxycytidine as an internal standard for 2'-deoxycytidine also allows the assessment of the amount of 5-methyl-2'-deoxycytidine relative to the total amount of cytosine residues. This method provides quantitative DNA methylation analysis that is more reliable and efficient than previous methods.

Applications of DNA Methylation Detection

[0020] The amount or degree of methylation of genomic DNA has implications in many conditions such as aging, genetic abnormalities, cancer and other disease states including atherosclerosis [41], Angelman syndrome [42], Duchenne muscular dystrophy [43] and ICF syndrome [44], to name a few. Aberrant CpG island-hypermethylation, which occurs at high frequency in tumors, can yield diagnostic information. Determining a patient's genome methylation by means of the present invention opens the way, in a cost-effective manner, for an unprecedented early warning diagnosis of many common cancers.

[0021] Knowledge gained, in accordance with the present invention, about cellular methylation fingerprints specific to a given disease also can illuminate improved therapeutic strategies. By combining disease-specific gene methylation analysis with information on global genomic methylation, one can establish which genes are being silenced, leading to acceleration of pathogenesis. Other applications for the present invention are detailed below.

[0022] Basic scientific research: The study of DNA methylation status is critical in many fields of basic scientific research. DNA methylation is a good biomarker of DNA metabolism and the assessment of DNA methylation is crucial to investigate DNA metabolic pathways such as DNA synthesis and repair. DNA methylation analysis also provides information on genomic stability and gene expression. DNA methylation information can be used to study the interaction between nutrients and genes in determining DNA methylation. For example, DNA methylation is a marker of one-carbon metabolism, a biochemical pathway that uses methyl-groups derived from the nutrient folate for several DNA metabolic reactions. Assessment of DNA methylation is fundamental to the understanding of the molecular basis of a large number of diseases in which epigenetic control of DNA is involved such as cancer, degenerative diseases, neurodevelopmental disorders or others. The methods described herein will enable basic researchers to further probe the relationship between DNA methylation and disease.

[0023] Diagnosis: Inappropriate methylation changes in CpG islands is one of the earliest known stages in the development of many cancers and direct detection of these molecular aberrations using the methods disclosed herein provides an

extraordinary opportunity for unprecedented early stage molecular diagnosis of cancer.

[0024] Enabling technology for improved clinical trials: The methods disclosed herein can be used to determine which individuals are afflicted with methylation dependent cancers. This can increase the success of efficacy studies in clinical trials of drugs targeting the basis for the methylation difference in the cancer. Drug candidates that effect methylation status represent the next generation of non-cytotoxic, cancer therapies.

[0025] Personalized Medicine: With the high cost of cancer therapies and the wide variety of cancer types, it is important that tests be developed to determine which patients will respond to which therapies. As the new generation of methylation dependent cancer therapies advances, the assays of the present invention will be important to determine which cancer patients are afflicted with methylation defects. Such data can help the oncologist's therapy decision process, determining patient suitability for a methylation-based drug regimen.

[0026] Discovery: Detection of inappropriate methylation in CpG islands acts as an indicator of which genes are involved in the development of cancer. Furthermore, only a small fraction (< 3 %) of all CpG islands have been investigated as to their role in cancer. Discoveries of new gene silencing events in cancer using the methods described herein will provide critical information for the initiation of new drug development strategies.

[0027] Toxicology: The cost of bringing drug candidates through clinical trials that eventually fail due to toxicological problems is enormous. Thus, there is a greater need for methylation detection methods to "weed-out" drugs with toxicology problems at an early (pre-clinical) stage. Applying the methods of the present invention in a high throughput screening format will be helpful in determining if a particular drug impacts the methylation status of cells or tissues. Such screening would lower the likelihood that candidate drugs with mutagentic or epigenetic-based toxicity will proceed inappropriately to clinical trials.

[0028] Thus, detecting methylation status by LC/MS/ESI can advance any purpose for which determination of methylation status is important. For instance, the present invention can be employed to determine whether a gene is involved in a

pathology, by determining the activation state of the gene based on its extent of cytosine CpG methylation.

BRIEF DESCRIPTION OF THE FIGURES

[0029] Figure 1 shows a chemical structure 2'-deoxycytidine and 5-methyl-2'-deoxycytidine, respectively. Cytosine base isotope-labeled compounds can be created by substituting any N with ^{4}N or ^{15}N, O may be ^{16}O or ^{18}O, C may be ^{13}C or ^{14}C and H may be ^{1}H or ^{2}H, in any combination.

[0030] Figure 2 presents a typical LC/MS chromatogram of DNA digests. In the top panel is represented the UV chromatogram obtained after complete enzymatic hydrolysis of human PBMC DNA and detected at 254 nm. The first peak eluting after 4.5 ± 0.5 min corresponded to cytosine (Cyt) and the second peak eluting after 6.5 ± 0.5 min corresponded to 5-methylcytosine (mCyt). In the middle panel is represented a typical MS chromatogram. The first two MS peaks corresponded to Cyt (m/z = 112) and to its stable isotope (m/z = 115) and the second two peaks corresponded to mCyt (m/z = 126) and its stable isotope (m/z = 130) as indicated in the bottom panel.

[0031] Figure 3 shows a DNA methylation status expressed as an absolute amount of 5-methylcytosine (ng mCyt/μg DNA) in samples of scalar concentrations of universal methylated human DNA. P for trend = 0.018 with 0.98 of Pearson's correlation coefficient.

[0032] Figure 4 demonstrates the correlation between DNA methylation and levels of plasma folate divided into tertiles and according by *MTHFR* genotype: *C/C* (left panel) and *T/T* (right panel).

[0033] Figure 5 demonstrates a correlation between DNA methylation and levels of different co-enzymatic forms of folate in *MTHFR T/T* subjects. An inverse relationship was detected between DNA methylation (expressed in log-scale) and formylated tetrahydrofolate polyglutamates (formyl-THF, left panel). A positive correlation was detected between DNA methylation and methyltetrahydrofolate polyglutamates (methyl-THF, right panel).

[0034] Figure 6 shows a simplified representation of the LC/MS method for the assessment of DNA methylation status. After the separation of DNA bases, the ESI conditions caused the separation of the pentose moiety from the pyrimidine ring of both 2'-deoxycytidine (WT= 227.1) and 5-methyl-2'-deoxycytidine (WT=241.2)

and resulted in the production of cytosine (WT=111.10) and 5-methylcytosine (WT=125.13), respectively. The molecular weight of the final fragment ions was consistent with the addition of a proton to the pyrimidines ring of both DNA bases (cytosine, $m/z = 112$; 5-methylcytosine $m/z = 126$).

EXAMPLES

[0035] Experiments were performed using a Hewlett Packard/Bruker Esquire-LC Ion Trap Liquid Chromatograph/Mass Spectrometer (Billerica, MA). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1315A). A Suplex pKb 100 analytical column (25 cm x 2.1 mm) protected by a 5 μm Suplex pKb 100 pre-column (2 cm x 2.1 mm) (Supelco, Bellefonte, PA, USA) was used. The HPLC system was controlled by an HP ChemStation software. The mass spectrometer, from Bruker Daltonik (Bremen, Germany), was equipped with an electrospray ionization (ESI) source. The mass spectrometer, system was controlled by an Esquire-LC NT™ software version 4.0. Both software packages run on an HP Kayak XA PC under Microsoft Windows NT™ version 4.0 operating system.

[0036] The mobile phase consisted of 7 mM ammonium acetate pH 6.7/methanol 5% (v/v) and was prepared using HPLC-grade purity water, methanol (both from J.T. Baker, Philipsburg, NJ), and ammonium acetate (Aldrich, Milwakee, WI). The mobile phase was filtered through a 0.2 μm nylon membrane filter (Alltech, Deerfield, IL) before use. The stable isotope-labeled compounds $^{15}N_3$ 2'-deoxycytidine and the custom-made methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine (both from Cambridge Isotopes Laboratories, Inc., Andover, MA) were used as internal standards for 2'-deoxycytidine and 5-methyl-2'-deoxycytidine residues, respectively. Quality control data showed a chemical purity of 98% for $^{15}N_3$ 2'-deoxycytidine and 95%+ for methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine and a 98%+ isotopic enrichment for both compounds.

[0037] Mixtures of 5-methyl-2'-deoxycytidine and 2'-deoxycytidine (Sigma, St. Louis, MO) were used as external standards, to create a calibration curve, to

evaluate the sensitivity of the technique for these compounds and to determine the limit of detection.

[0038] CpGenome™ universal enzymatically methylated human male genomic DNA was used (Intergen Company, Purchase, NY) to evaluate different amounts of 5-methyl-2'-deoxycytidine in human DNA.

[0039] Genomic DNA was extracted from the buffy coat of human blood using a classical phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] protocol. Residual RNA was treated with both RNase A and T_1 (Invitrogen, Carlsbad, CA) to a final concentration of 10 units/ml at 37°C for 1 hour. The DNA was re-precipitated with 7.5 M ammonium acetate (1:1/2, v/v) and ethanol 100% (1:2, v/v) and dissolved in TE buffer (10mM tris HCl, 1mM EDTA pH 8.0). DNA was dialyzed on a 0.025 μm filter paper (Millipore, Bedford, MA) and stored at -70°C until analysis. The procedure of DNA hydrolysis was performed as described by Crain.[33] Briefly, 1 μg of DNA was denatured by heating at 100°C for 3 min and subsequently chilled in ice slush. One-tenth volume of 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1 (Roche Molecular Biochemicals, Mannheim, Germany) were added. The mixture was then incubated at 45°C for 2 hours. To the solution were subsequently added 1/10 volume of 1 M ammonium bicarbonate (Sigma, St. Louis, MO) and 0.002 units of venom phosphodiesterase I (Sigma, St. Louis, MO). The incubation was continued for an additional 2 hours at 37°C. Thereafter, the mixture was incubated for 1 hour at 37°C with 0.5 units alkaline phosphatase (Sigma, St. Louis, MO). The stable isotopes $^{15}N_3$ 2'-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine were then added to the samples to reach a final concentration of 1 ng/μl and 0.5 ng/μl, respectively, in a total volume of 35 μl.

[0040] LC/ESI/MS procedure. Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The DAD was calibrated and tested using the DAD diagnosis procedure of the ChemStation software for HP1100 system. The HP1100 MSD was calibrated with ESI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range.

[0041] Twenty µl of the hydrolyzed-DNA solution were injected onto the analytical column thermostated at 21°C. The separation of the four major DNA bases as well as that of 5-methyl-2'-deoxycytidine was obtained by isocratic elution. The mobile phase flow rate was 0.3 ml/min and the run time for 13 minutes. Electrospray source conditions were: capillary 30 nA, nitrogen drying gas 9.0 l/min, with auxiliary 40.0 psi gas to assist with nebulization and drying temperature of 350°C. The mass spectrometer was operated at a capillary voltage of 2500 V and spectra were collected in positive ion mode. The electrospray needle was maintained at ground. The ion trap mass range was set to evaluate 2'-deoxycytidine from *m/z* 110 to *m/z* 118 and 5-methyl-2'-deoxycytidine from *m/z* 125 to *m/z* 132 in two different time windows. The time window for 2'-deoxycytidine was from 0 to 5.5 minutes, and the time run window for 5-methyl-2'-deoxycytidine was from 5.5 to 13 minutes (shown in Fig.1). The acquisitions were typically 16 time points per minute. Sixty scans were obtained per time point and they were averaged before being stored in the database. Identification of 2'-deoxycytidine and 5-methyl-2'-deoxycytidine was obtained by combined UV detection at 254 nm and 280 nm, and MS analysis of the chromatographic peaks eluting after 4.5 ± 0.5 min and 6.5 ± 0.5 min, respectively.

[0042] The separation of the four bases and 5-methyl-2'-deoxycytidine residues was obtained using a reverse-phase column as described by Zambonin et al.[31] This method was modified using isocratic elution of a mobile phase that consisted of 7 mM ammonium acetate pH 6.7/5%methanol. The time run was 13 min.

[0043] As shown in Scheme 1, the electrospray ionization conditions caused the loss of the 2'-deoxyribose moiety in both 2'-deoxycytidine and 5-methyl-2'-deoxycytidine and the production of fragment ions whose molecular weight was consistent with the addition of a proton to the pyrimidine ring for both DNA bases (*m/z* 112 and *m/z* 126, respectively).

[0044] As expected, the ESI source, with the mass spectrometer in positive ion detection mode, gave protonated molecules and also fragment ions for both 2'-deoxycytidine and 5-methyl-2'-deoxycytidine. We chose to monitor the fragment ions of the compounds of interest rather than the protonated molecules because they provided more signal and better quantification results.

[0045] Figure 2 shows a typical LC/MS chromatogram for a sample of purified and completely hydrolyzed peripheral blood mononuclear cell (PBMC) DNA. The top panel shows a UV chromatogram obtained at 254 nm. The middle panel shows a set of mass chromatograms. The HPLC peak eluting after 4.5 ± 0.5 min and at *m/z* 112 corresponded to 2'-deoxycytidine and the HPLC peak eluting after 6.5 ± 0.5 min, and at *m/z* 126 corresponded to 5-methyl-2'-deoxycytidine.

[0046] The UV chromatogram was used to verify the complete digestion of the DNA samples as well as the absence of RNA residues. As previously described by Crain et al., [26] an incomplete digestion of DNA due the aberrant function of one or more enzymes could adversely affect the results particularly in view of the very small amounts of 5-methyl-2'-deoxycytidine present in DNA.[33] The presence of RNA can interfere with the determination of the methylated cytosine residues in DNA since both tRNA and rRNA contain 5-methylcytidine.[45,46] Because of the loss of the pentose moiety during the ESI procedure, the attribution of 5-methylcytosine exclusively to DNA is not possible. Therefore, we attempted to minimize the possible RNA contamination by using two different RNases and cleaning up the DNA by re-precipitation. In the case of RNA contamination, the chromatogram shows the presence of an extra peak eluting at 3.5 ± 0.5 min. The ESI source with the mass spectrometer in positive ion detection mode, gave protonated molecules as well as fragment ions for both 2'-deoxycytidine and 5-methyl-2'-deoxycytidine, whose molecular weight was consistent with the addition of a proton to the pyrimidine ring for both DNA bases (Figure 2). The molecular weight of the molecule eluting at 3.5 ± 0.5 min was consistent with the addition of a proton to the cytidine residue (*m/z* 244). All the DNA samples were treated to avoid the presence of residual RNA, as described above, and no extra peaks were detected.

[0047] As shown in Figure 2, the ion at *m/z* 115 corresponded to the isotope labeled $^{15}N_3$ 2'-deoxycytidine and the ion at *m/z* 130 corresponded to the isotope labeled methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine. The signal to noise ratio for a 20 µl injection of a 5 pg/µl concentrated sample of 2'-deoxycytidine and 5-methyl-2'-deoxycytidine, was 8.5 and 12.4, respectively.

[0048] The correlation between the MS signal and concentrations of 2'-deoxycytidine and 5-methyl-2'-deoxycytidine was linear for scalar concentrations of

both external standards ranging from 0.1 ng/μl to 10 ng/μl, the usual working range. The Pearson's correlation coefficient was 0.991 for 2'-deoxycytidine and 0.994 for 5-methyl-2'-deoxycytidine.

[0049] The ratios between each of the external standards and the corresponding stable isotopomer remained constant, when scalar concentration of the external standards for 2'-deoxycytidine and 5-methyl-2'-deoxycytidine were compared with a fixed concentration of the corresponding stable isotopomer. The concentrations of 2'-deoxycytidine ranged from 0.0175 ng/μl to 3.5 ng/μl, and the concentration of $^{15}N_3$ 2'-deoxycytidine was 3.5 ng/μl. The Pearson's correlation coefficient of expected and observed ratios between scalar concentrations of 2'-deoxycytidine and its isotopomer $^{15}N_3$ 2'-deoxycytidine was 0.999 with a $P = 0.002$

[0050] The correlation of expected and observed ratios between scalar concentration of the external standard 5-methyl-2'-deoxycytidine (from 0.0175 ng/μl to 3.5 ng/μl) and fixed concentrations of its corresponding isotope labeled methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine (1.75 ng//μl) showed a Pearson's correlation coefficient of 0.999 and a P-value < 0.0001. The concentration of the stable isotopes was chosen for both internal standards on the basis of values obtained from a series of experiments from biological samples that gave the best percent relative standard deviation (usually < 5%). These concentrations were 3.5 ng/μl for $^{15}N_3$ 2'-deoxycytidine and 1.75 ng/μl for methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine.

[0051] With this method, DNA methylation status can be expressed either by the absolute amount of 5-methyl-2'-deoxycytidine (i.e. ng/μg DNA) or by the relative amount of 5-methyl-2'-deoxycytidine compared to total cytosine residues (i.e. % of total cytosines).

[0052] The absolute amount of 5-methyl-2'-deoxycytidine per μg DNA was calculated using the abundance ratio between the 5-methyl-2'-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine. To express the amount of 5-methyl-2'-deoxycytidine as a percentage the following equation was used: 5-methyl-2'-deoxycytidine/(5-methyl-2'-deoxycytidine + 2'-deoxycytidine) x 100.

[0053] To determine the precision of the method, we performed a series of experiments using PBMC DNA samples. For quantification of both 2'-deoxycytidine

and 5-methyl-2'-deoxycytidine we compared the values obtained from biological samples with those of the internal standards of known concentration.

[0054] To determine the within-day percent relative standard deviation (%RSD) we ran nine samples of DNA hydrolyzates from the same DNA aliquot. As shown in Table 1 the within-day %RSD for data expressed as the absolute amount of 5-methyl-2'-deoxycytidine was 7.1. In Table 1 is shown the within-day %RSD for data expressed as 5-methyl-2'-deoxycytidine percentage of total 2'-deoxycytidines (1.9).

[0055] To evaluate the precision of an experiment using the same DNA sample performed in different days, nine aliquots from the same human PBMC DNA sample were run for three days in a row. As shown in Table 1 the day-to-day %RSD was 5.7 for data expressed as absolute amount of 5-methyl-2'-deoxycytidine. The day-to-day %RSD was 1.7 for data expressed as 5-methylcytosine percentage of total cytosines.

[0056] As shown in Table 1 the proportion of methylcytosine residues in human genomic DNA was around 5%, which is similar to that previously described by others.[1]

[0057] To verify the method's ability to detect different quantities of 5-methyl-2'-deoxycytidine in a proportional manner we also performed experiments with scalar amounts of universal methylated human genomic DNA as represented in Figure 3. There was a linear correlation between concentration of universal methylated DNA and absolute amount of 5-methyl-2'-deoxycytidine ($r = 0.98$, P for trend = 0.018).

[0058] The present inventors have used this method to analyze the effect of folate status on genomic DNA methylation. The inventors investigated the interaction between folate status, DNA methylation, and the common *C677T* mutation in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene [47]. The present invention was used to assess genomic DNA methylation in peripheral blood mononuclear cell DNA from 105 subjects homozygous for this mutation (*T/T*) and 187 homozygous for the wild-type (*C/C*) *MTHFR* genotype. The results show that genomic DNA methylation directly correlates with folate status and inversely with plasma homocysteine (tHcy) levels ($P<0.01$). *T/T* genotypes had a diminished level of DNA

methylation compared to those with the *C/C* wild-type (32.23 *vs.*62.24 ng 5-methylcytosine/μg DNA, $P<0.0001$). When analyzed according to folate status, however, only the *T/T* subjects with low levels of folate accounted for the diminished DNA methylation ($P<0.0001$). Moreover, in *T/T* subjects DNA methylation status correlated with the methylated proportion of red blood cell folate (RBC) and was inversely related to the formylated proportion of RBC folates ($P<0.03$) that is known to be solely represented in those individuals.

Materials and Methods

Study population

[0059] A total of 292 unrelated, age- and sex-matched subjects recruited from a single geographical area (northern Italy) were studied. Characteristics of the study population including *MTHFR* genotype distribution are described in detail elsewhere [48,49]. Of these 292 subjects, 193 had angiographically documented coronary atherosclerosis. Ninety-nine had normal coronary arteries as documented by angiography and had neither clinical nor laboratory evidence of atherosclerosis. They were examined for reasons other than coronary artery disease (in most cases valvular heart disease) [48,49]. Informed consent was obtained from all subjects after a full explanation of the study. The University Hospital of Verona Review Board approved the study.

Biochemical Analysis

[0060] Samples of venous blood were drawn from each subject after an over-night fast. DNA was extracted from whole blood using a phenol:chloroform:isoamyl alcohol protocol. All the laboratory methods for biochemical analyses have been performed as described elsewhere [48]. tHcy levels were measured by high-performance liquid chromatography (HPLC) with fluorescent detection according to Araki and Sako [50]. Plasma folate, and vitamin B_{12} concentrations were measured by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA). Vitamin B_6 levels were measured by HPLC method according to Kimura M, et al.[51]. RBC folate form distribution was detected by affinity followed by reverse-phase chromatography with electrochemical detection as previously described[52,53].

MTHFR genotyping

[0061] The analysis of the *MTHFR C677T* mutation was performed by PCR followed by *Hinf* I digestion according to Frosst et al. [54].

DNA methylation measurement

[0062] The present LC/MS method was used to measure genomic DNA methylation. The instrument was a HP-Bruker Esquire, LC Ion Trap LC/MS (Billerica, MA) equipped with an electrospray ionization source. One μg DNA was enzymatically hydrolyzed by sequential digestion with 2 units nuclease P1, 0.002 units of venom phosphodiesterase I, and 0.5 units alkaline phosphatase as described by Crain [33]. Twenty μl of the hydrolyzed-DNA solution was injected onto a Suplex pKb 100 analytical column (250 x 2.1 mm i.d.) protected by a 5 μm Suplex pKb 100 pre-column (Supelco, Bellefonte, PA, USA) [31]. The isocratic mobile phase consisted of 7 mM ammonium acetate pH 6.7/methanol (5% v/v) in water delivered at a flow rate of 0.3 ml/min for 13 minutes. This allowed the separation of the four DNA bases as well as the identification of 5-methylcytosine. Mass spectrometry operating conditions relating to the electrospray source were as follows: capillary 30 nA, nebulizer 40.0 psi, N_2 drying gas 9 l/min and drying temperature 350° C. The ion trap was scanned from *m/z* 100 to 150. 2'-deoxycytidine (U-$^{15}N_3$) (Cambridge Isotopes Laboratories, Inc., Andover, MA) was used as an internal standard and mixtures of 5-methyl-2'-deoxycytidine and 2'-deoxycytidine (Sigma, St. Louis, MO) were used as external standards. Identification of cytosine (Cyt) and 5-methylcytosine (mCyt) was obtained by combined UV detection at 254 nm and MS analysis of the chromatographic peaks eluting after 4.5 ± 0.5 min and 6.5 ± 0.5 min, respectively. The ion peak at $m/z = 112$ corresponded to Cyt and the ion peak at $m/z = 126$ corresponded to mCyt, confirming what was described also by others [32]. The ion peak at $m/z = 115$ corresponded to the stable isotope used as internal standard. Quantification of Cyt was done by comparison to its internal standard and mCyt quantification was obtained by comparison with external standards of known concentration since a stable isotope standard for mCyt was not commercially available. The linear working range of the method was from 100 pg to 10 μg. The limit of detection for both Cyt and mCyt was 100 pg. DNA methylation status was

defined as the amount of mCyt per μg of DNA. Coefficients of variance (n = 5) were 1.6% (within day) and 5.7% (between days).

Statistical Analysis

[0063] All computations were performed by using the Systat software10.0 program for Windows (2000, SPSS Inc., Chicago, IL). The distribution of continuous variables in groups are expressed as mean ± SD. Analysis was performed with log-transformed data for all skewed variables including DNA methylation. Therefore, geometric means (antilogarithms of the transformed means) are presented and 95% confidence intervals (CIs) are calculated using the transformed values and displayed as the antilogarithms of the transformed data. Statistical significance for differences in continuous variables was tested by Student's unpaired t-test. Categorical variables were analyzed using a chi-squared test or linear regression analysis when appropriate. Adjustment for confounding variables (*i.e.* age, sex, smoking, creatinine and vitamin status when appropriate) was performed by general linear model analysis (specifically analysis of covariance). Statistical significance refers to a two-tailed analysis where $P<0.05$.

Results

[0064] A linear regression analysis, pooling both genotypes, demonstrated highly significant direct relationships between plasma and RBC folate and DNA methylation ($P<0.01$). However, such relationships were driven entirely by the data from the *T/T* individuals and were not significant among the *C/C* individuals (*vide infra*). An indirect relationship was observed between DNA methylation and tHcy ($P<0.01$). We found no significant correlation between DNA methylation and vitamin B_6 or vitamin B_{12} status.

[0065] The characteristics of the population according to *MTHFR* genotype are described in Table 2. As shown, no differences were found in age, sex, vitamin B_6 and vitamin B_{12} levels between the *MTHFR C677T* wild-type (*C/C*) and the homozygous mutant (*T/T*) groups. Plasma folate, as well the RBC folate concentrations were lower in the *T/T* as compared to the *C/C* group ($P<0.0001$). Total plasma homocysteine was higher in the *T/T* than in the *C/C* group ($P<0.0001$). These differences between the two *MTHFR* genotypes also extend to DNA methylation. The

mean level of mCyt in the DNA from the *T/T* group was approximately half of that found in the *C/C* group ($P<0.0001$) (Table 2). This difference between genotypes is most evident when folate status was taken into account. Among those whose plasma folate status is above the median value (12 nmol/L), genomic DNA methylation was similar in the two *MTHFR* genotypes (Table 3). However, when plasma folate status was below the median, the level of DNA methylation was considerably lower among the subjects homozygous for the *C677T* mutation than for the wild-type genotype. Moreover, the level of genomic DNA methylation was a 50% lower in this group compared to other *T/T* individuals ($P<0.0001$) with levels of folate equal or above the median or with the *C/C* group in the lower range of plasma folate (Table 3). Further illustration of these differences is shown in Figure 3. When the population sample was divided into tertiles of plasma folate according to *C/C* and *T/T* genotype, a graded effect of folate in determining levels of DNA methylation was detected only among the *T/T* individuals. The level of DNA methylation among those *T/T* individuals in the lowest tertile of folate compared to the highest was approximately a 35% lower and was statistically significant ($P<0.03$) (Figure 4). Among those with the *C/C* genotype, however, DNA methylation was not different across the tertiles.

[0066] The results of the analyses when RBC folate was used as a measure of folate status precisely paralleled the observations made with plasma folate (Table 4). When both the *C/C* and the *T/T* individuals are divided into groups according to the median RBC folate value (1.13 nmol/folate/g Hb), only the *T/T* group with folate below the median had diminished DNA methylation ($P<0.0001$). No such differences were found in the *C/C* group (Table 4). At RBC folate levels above the median, DNA methylation was not influenced by the *MTHFR* genotype.

[0067] The assessment of RBC folate vitamer distribution demonstrated that the folate contained in RBCs from individuals carrying the *C/C* genotype is comprised entirely of 5-methyl tetrahydrofolates, whereas the RBC folate from individuals with the homozygous mutant *T/T* genotype consists of 30 % formylated THF polyglutamates confirming what we previously described [52]. Furthermore, the profile of folate co-enzymes within the cell of *T/T* individuals bears a close relationship with peripheral blood mononuclear cell DNA methylation as shown in Figure 5: a regression analysis within the *T/T* group, revealed an inverse relationship between the

proportion of formyl-THF and DNA methylation ($P<0.03$) as well as a positive association between DNA methylation and methyl-THF proportions ($P<0.03$).

[0068] These results indicate that the *MTHFR C677T* polymorphism influences DNA methylation status through an interaction with folate status. This example demonstrates the efficiency at which a large number of samples can be analyzed using the present method.

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[0069] The references cited herein and throughout the specification are herein incorporated by reference in their entirety.

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Table 1. Precision of the LC/MS method for the determination of 5-methyl-2'-deoxycytidine

Within-day variation (n=9)

Absolute amount of 5-methyl-2'-deoxycytidine[a] (ng mCyt/μg DNA)	RSD[b] (%)	Percentage of 5-methyl-2'-deoxycytidine[a] (%)[c]	RSD[b] (%)
4.8±0.3	7.1	5.8±0.1	1.9

Day-to-day variation (n=9)

Absolute amount of 5-methyl-2'-deoxycytidine[a] (ng mCyt/μg DNA)	RSD[b] (%)	Percentage of 5-methyl-2'-deoxycytidine[a] (%)[c]	RSD[b] (%)
4.3±0.2	5.7	5.4±0.1	1.7

[a] From samples of human PBMC DNA. Values are expressed as means ± SD.
[b] RSD: Relative standard deviation.
[c] Equation to calculate the percentage of 5-methyl-2'-deoxycytidine (mCyt) of the total 2'-deoxycytidine (Cyt) = (mCyt/mCyt +Cyt) x100.

Table 2. Characteristics of the population according to *MTHFR* genotype.

	C/C	*T/T*	*P*-value
	N=187	N=105	
Age	59.77±9.96	59.92±11.39	N.S.
Sex, % male	80.21	77.14	N.S.*
Plasma folate (nmol/L)	12.71 (9.81-11.96)	10.42 (9.58-11.33)	<0.0001
RBC folate (nmol/g Hb)	1267.49±684.28	833.55±435.84	<0.0001
Vitamin B_{12} (pmol/L)	297 (280-315)	292 (274-316)	N.S.
Vitamin B_6 (nmol//L)	30.38 (27.93-33.04)	33.85 (30.17-37.97)	N.S.
tHcy (μmol/L)	14.70 (13.90-15.54)	21.26 (19.64-22.98)	<0.0001
Genomic DNA methylation status (ng mCyt/μg DNA)	62.24 (54.32-71.31)	32.23 (24.78-41.92)	<0.0001

Values are expressed as mean ± SD for age and RBC folate. Plasma folate, vitamin B_{12}, vitamin B_6, tHcy and DNA methylation status are presented as geometric means (antilogarithms of the transformed means) and 95% confidence intervals are reported in parentheses with 2-tailed P-values.
Statistical difference was evaluated by Student's t-test except when differently indicated.
* Statistical difference was evaluated by χ^2 test. N.S. = not statistically significant.

Table 3. Genomic DNA methylation status (ng mCyt/μg DNA) according to *MTHFR* genotype and plasma folate levels (stratification by folate values above and below the median).

	Plasma folate ≥ 12 nmol/L	**Plasma folate ≤ 12 nmol/L**	***P*-value**
C/C N=187	64.00 (52.72-77.63)	63.05 (51.47-77.25)	N.S.
T/T N=105	67.56 (50.45-90.38)	26.21 (18.81-36.49)	<0.0001
***P*-value**	N.S.	<0.0001	

DNA methylation status is presented as geometric mean (antilogarithm of the transformed means) and 95% confidence intervals are reported in parentheses with 2-tailed P-values. Statistical difference was evaluated by Student's t-test. N.S. = not statistically significant.

Table 4. RBCs folate forms distribution and DNA methylation status according to *MTHFR C677T* genotype and stratification in low and high RBCs folate values

	Low RBC folate			*High RBC folate*		
	C/C	*T/T*	*P*-value	*C/C*	*T/T*	*P*-value
Total folate (nmol/g Hb)	0.81±0.20	0.68±0.27	0.003	1.69±0.70	1.48±0.27	N.S.
RBC methyl-THF (% of total)	98.8±5.7	67.3±29.0	< 0.0001	99.4±1.1	69.6±30.9	0.002
Genomic DNA methylation status (ng mCyt/μg DNA)	64.07 (49.89-81.45)	21.93 (14.73-32.45)	< 0.0001	57.97 (45.60-73.55)	57.39 (29.96-109.94)	N.S.

Low and high RBCs folate status is intended for levels below and above the total RBCs median value (1.13 nmol/g hemoglobin), respectively. Values are expressed as mean ± SD for RBCs folate levels. DNA methylation is presented as geometric mean (antilogarithm of the transformed mean) and 95% confidence intervals are reported in parentheses with 2-tailed P-values. Statistical difference was evaluated by Student's t-test. N.S. = not statistically significant.

CLAIMS

We claim:

1. A method for measuring DNA methylation, comprising:

(a) isolating genomic DNA,

(b) hydrolyzing the genomic DNA to form DNA hydrolyzates,

(c) adding methylated and/or unmethylated stable cytosine base isotopes to the DNA hydrolyzates,

(d) subjecting the DNA hydrolyzates to reverse-phase high performance liquid chromatography,

(e) identifying the products in step (d) by electrospray ionization enabled mass spectrometry, and

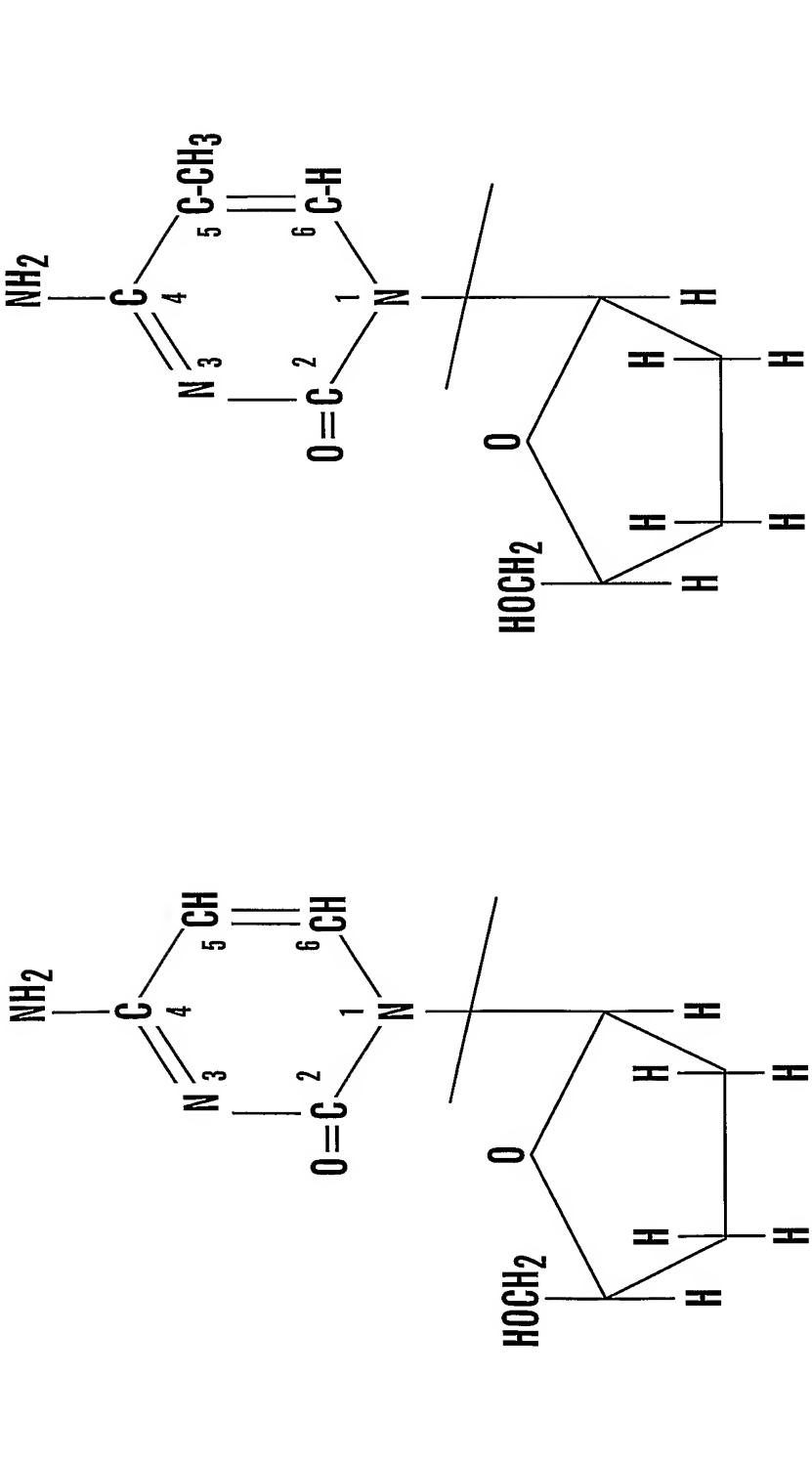
(f) quantitating DNA methylation.

2. The method of claim 1, wherein the stable isotopomer $^{15}N_3$ 2'-deoxycytidine is added as the internal standard for 2'-deoxycytidine, or wherein the stable isotopomer methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine is added as the internal standard for 5-methyl-2'-deoxycytidine.

3. The method of claim 1 step (f), wherein DNA methylation is quantitated by equations:

absolute amount of 5- methyl-2'-deoxycytidine/ug DNA=abundance ratio between 5- methyl-2'-deoxycytidine and methyl-D3, ring-6-D1 5- methyl-2'-deoxycytidine; and

5- methyl-2'-deoxycytidine expressed as % total of cytosine residues= 5-methyl-'-deoxycytidine/(5- methyl-2'-deoxycytidine+2'-deoxycytidine)*100.



CHEMICAL STRUCTURE 2'-deoxycytidine AND 5-methyl-2'-deoxycytidine RESPECTIVELY. CYTOSINE BASE ISOTOPE-LABELED COMPOUNDS CAN BE CREATED BY SUBSTITUTING ANY N WITH ^{15}N, OR H WITH *d*, OR C WITH ^{14}C OR O WITH ^{18}O, IN ANY POSSIBLE COMBINATION.

FIG. 1

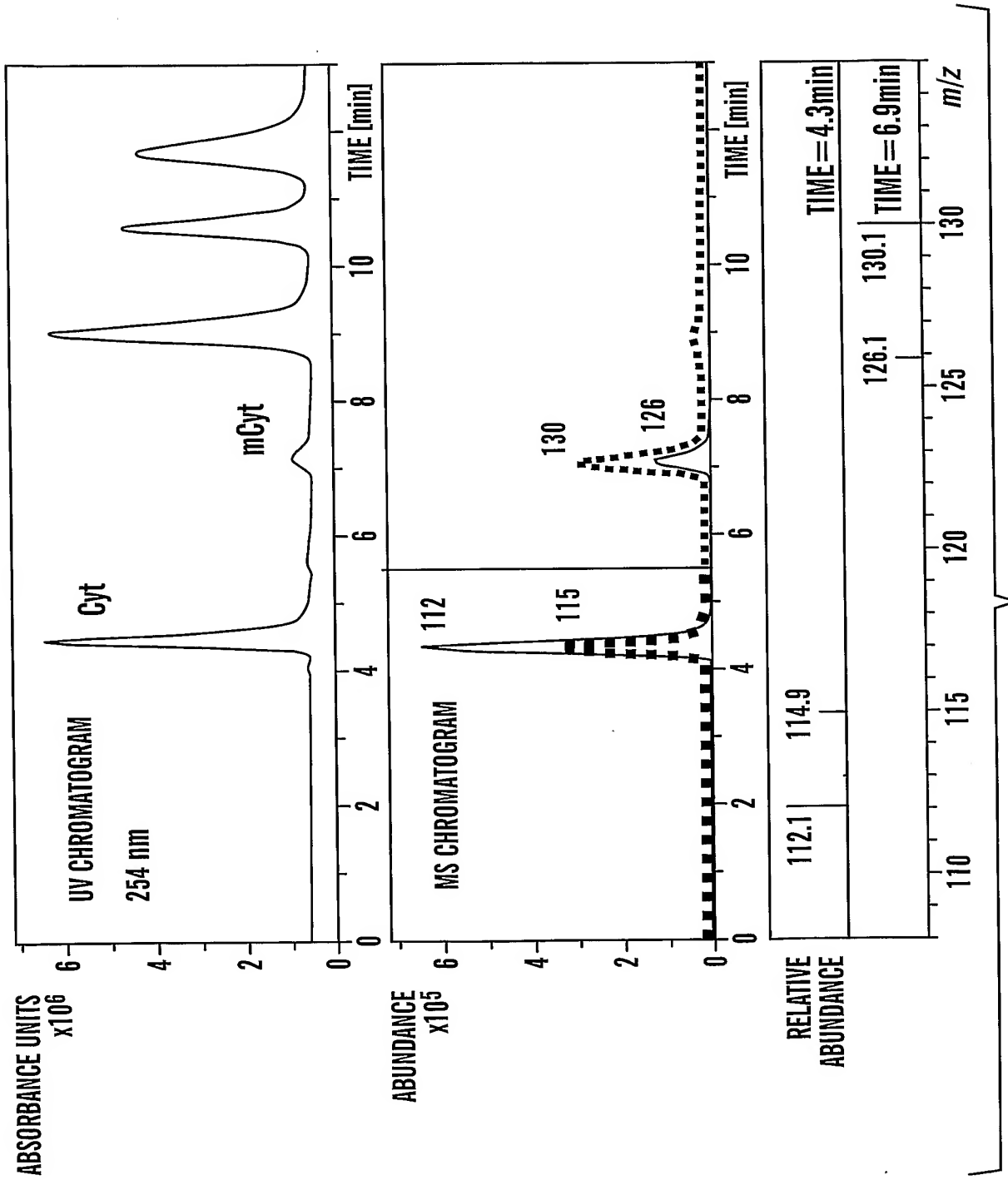

ABSORBANCE UNITS x10^6
UV CHROMATOGRAM
254 nm
Cyt
mCyt
TIME [min]
ABUNDANCE x10^5
MS CHROMATOGRAM
112
115
130
126
TIME [min]
RELATIVE ABUNDANCE
112.1
114.9
126.1
130.1
TIME=4.3min
TIME=6.9min
m/z

FIG. 2

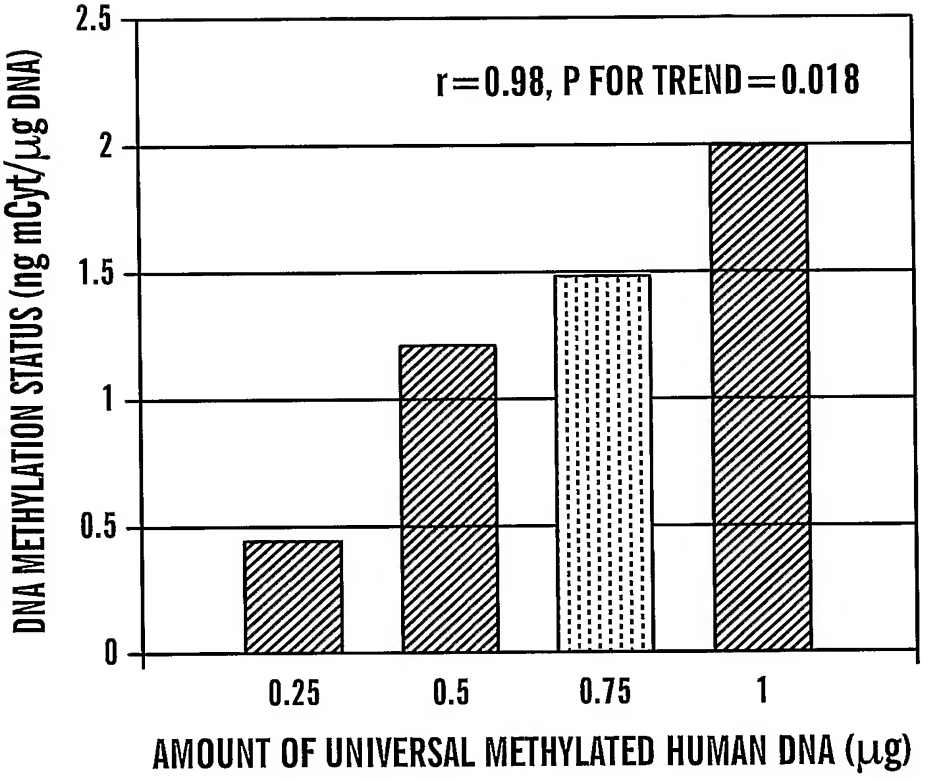

r=0.98, P FOR TREND=0.018
DNA METHYLATION STATUS (ng mCyt/μg DNA)
2.5
2
1.5
1
0.5
0
0.25
0.5
0.75
1
AMOUNT OF UNIVERSAL METHYLATED HUMAN DNA (μg)

FIG. 3

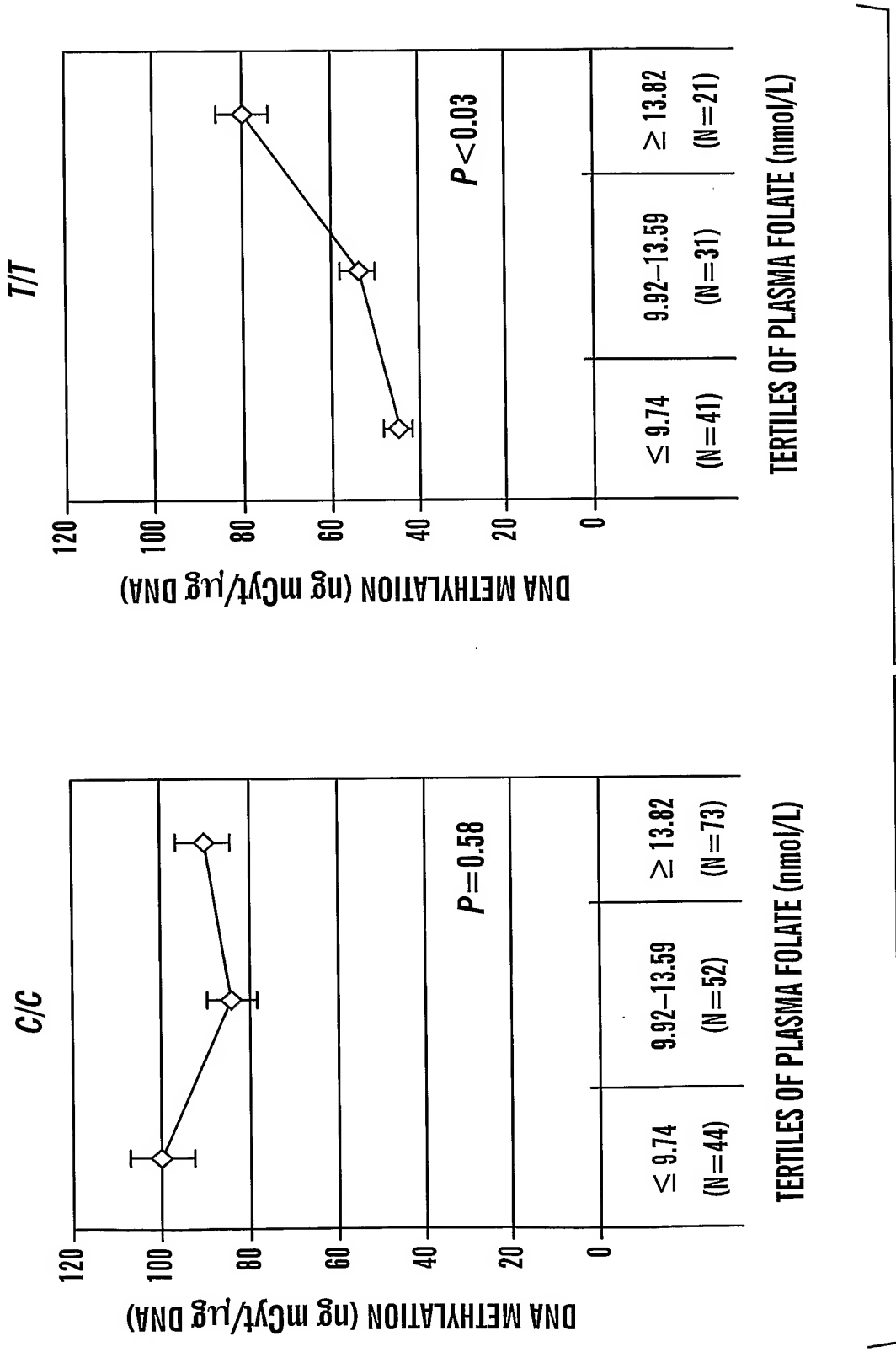

C/C
T/T
DNA METHYLATION (ng mCyt/μg DNA)
120
100
80
60
40
20
0
P=0.58
P<0.03
≤ 9.74 (N=44)
9.92–13.59 (N=52)
≥ 13.82 (N=73)
≤ 9.74 (N=41)
9.92–13.59 (N=31)
≥ 13.82 (N=21)
TERTILES OF PLASMA FOLATE (nmol/L)

FIG. 4

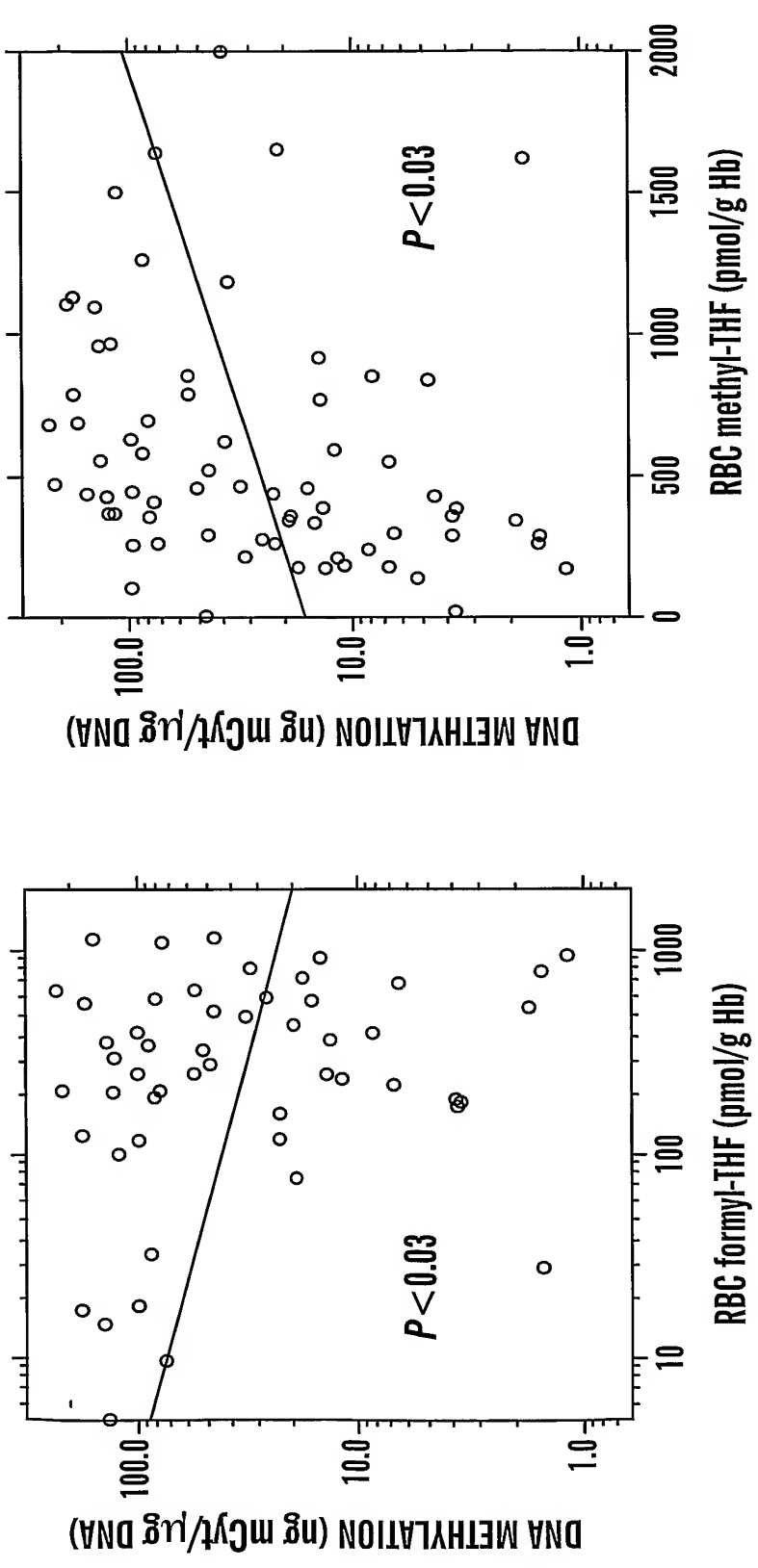

DNA METHYLATION (ng mCyt/μg DNA)
100.0
10.0
1.0
P<0.03
10
100
1000
RBC formyl-THF (pmol/g Hb)
DNA METHYLATION (ng mCyt/μg DNA)
100.0
10.0
1.0
P<0.03
0
500
1000
1500
2000
RBC methyl-THF (pmol/g Hb)

FIG. 5

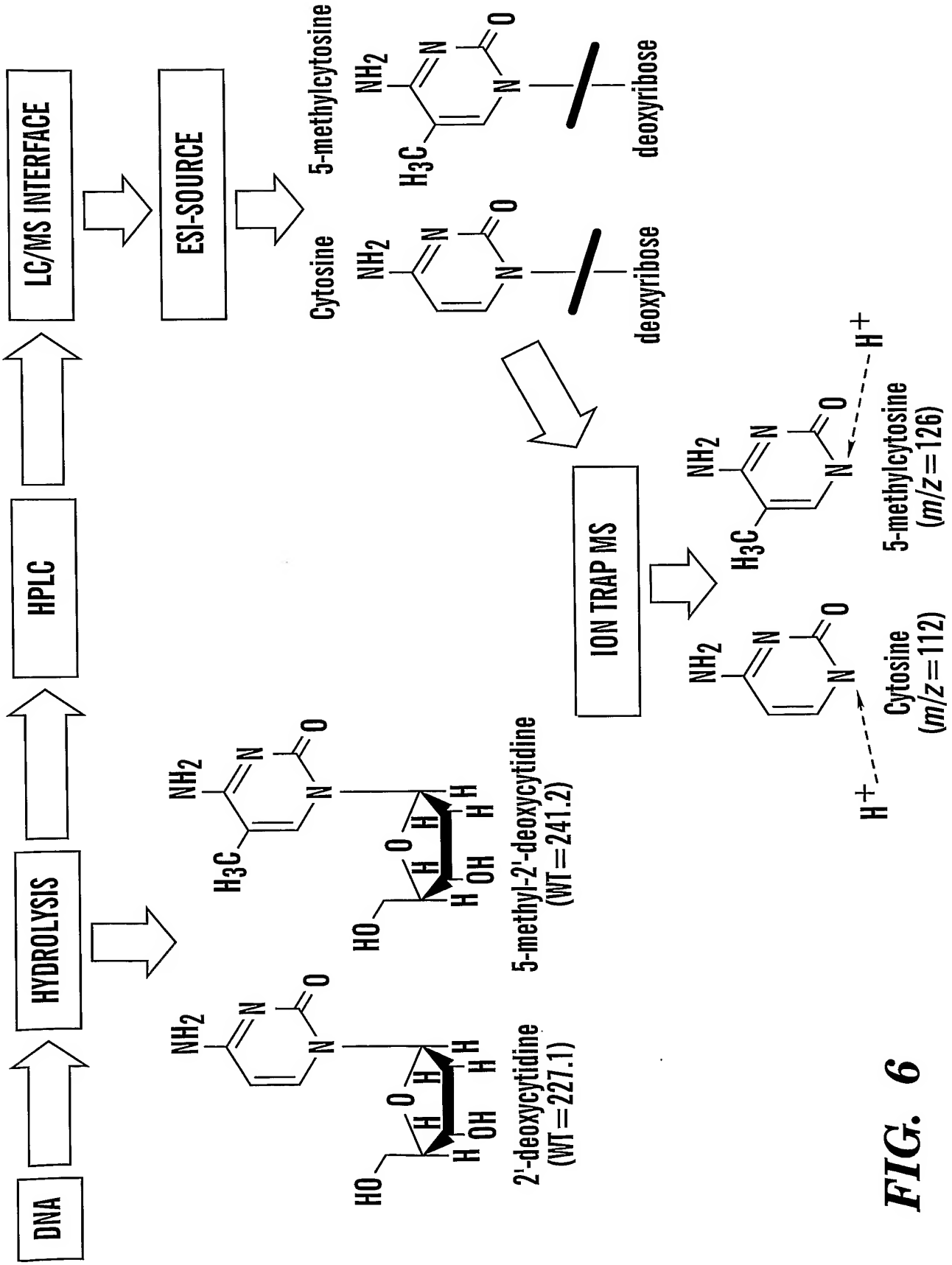

DNA
HYDROLYSIS
HPLC
LC/MS INTERFACE
ESI-SOURCE
Cytosine
5-methylcytosine
deoxyribose
deoxyribose
ION TRAP MS
2'-deoxycytidine
(WT=227.1)
5-methyl-2'-deoxycytidine
(WT=241.2)
Cytosine
(m/z=112)
5-methylcytosine
(m/z=126)

FIG. 6